REVIEW

Phosphoinositide-derived messengers in endocrine signaling

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Abstract

One of the fundamental questions in endocrinology is how circulating or locally produced hormones affect target cell functions by activating specific receptors linked to numerous signal-transduction pathways. An important subset of G protein-coupled cell-surface receptors can activate phospholipase C enzymes to hydrolyze a small but critically important class of phospholipids, the phosphoinositides. Although this signaling pathway has been extensively explored over the last 20 years, this has proven to be only the tip of the iceberg, and the multiplicity and diversity of the cellular functions controlled by phosphoinositides have surpassed any imagination. Phosphoinositides have been found to be key regulators of ion channels and transporters, and controllers of vesicular trafficking and the transport of lipids between intracellular membranes. Essentially, they organize the recruitment and regulation of signaling protein complexes in specific membrane compartments. While many of these processes have been classically studied by cell biologists, molecular endocrinology cannot ignore these recent advances, and now needs to integrate the cell biologist's views in the modern concept of how hormones affect cell functions and how derailment of simple molecular events can lead to complex endocrine and metabolic disorders.

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Introduction

The mechanism of action of 'Ca^{2+}-mobilizing hormones', a term introduced in the mid-1970s to denote hormones that exert their effects through generation of a cytosolic Ca^{2+} signal, was largely elucidated by the end of the 1980s (Fig. 1 and 2). The basic elements of this canonical signal-transduction cascade include the activation of the phospholipase C (PLC) β and γ isoforms by G protein-coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs) respectively. Stimulation of these receptors generates two second messengers, the soluble Ins(1,4,5)P_3 ($InsP_3$) and the hydrophobic diacylglycerol from membrane, PtdIns(4,5)P_2 (Berridge 1984). Once generated, the Ca^{2+}-mobilizing messenger, $InsP_3$, binds to specific receptors that are located mainly in the endoplasmic reticulum (ER) and function as tetrameric cation channels to release Ca^{2+} from intracellular Ca^{2+} stores (Mikoshiba 1997). This endogenous Ca^{2+} signal, in combination with increased Ca^{2+} influx via multiple Ca^{2+} entry mechanisms, activates numerous cytoplasmic and membrane-bound effector molecules with the help of Ca^{2+}-binding proteins. Ca^{2+} is also taken up by mitochondria, where it stimulates metabolic enzymes (Hajnoczky *et al.* 1995) or initiates complex responses such as apoptosis (Pacher & Hajnoczky 2001). The other limb of this messenger system, diacylglycerol (DAG), directly activates several members of the protein kinase C family (Nishizuka 1988), and can also contribute to the direct regulation of some ion channels (Hardie 2003).

This elegantly simple concept marked only the beginning of an amazing explosion of research into phosphoinositides, and almost every component of the calcium-phosphoinositide messenger system has grown to become a research field of its own. The metabolism of $InsP_3$ via both phosphorylation and sequential dephosphorylation revealed the identity of several inositol phosphate isomers (Shears 1998), as well as the InsP kinases (Verbsky *et al.* 2005) and phosphatases (Majerus *et al.* 1999). We are just at the beginning of understanding which of the numerous inositol phosphate isomers have regulatory functions. The discovery of phosphoinositide 3-kinases (PI3K) was the starting point in the recognition of the function of phosphoinositides as membrane-bound signaling molecules and was one of the main driving forces behind the search for proteins that are downstream targets

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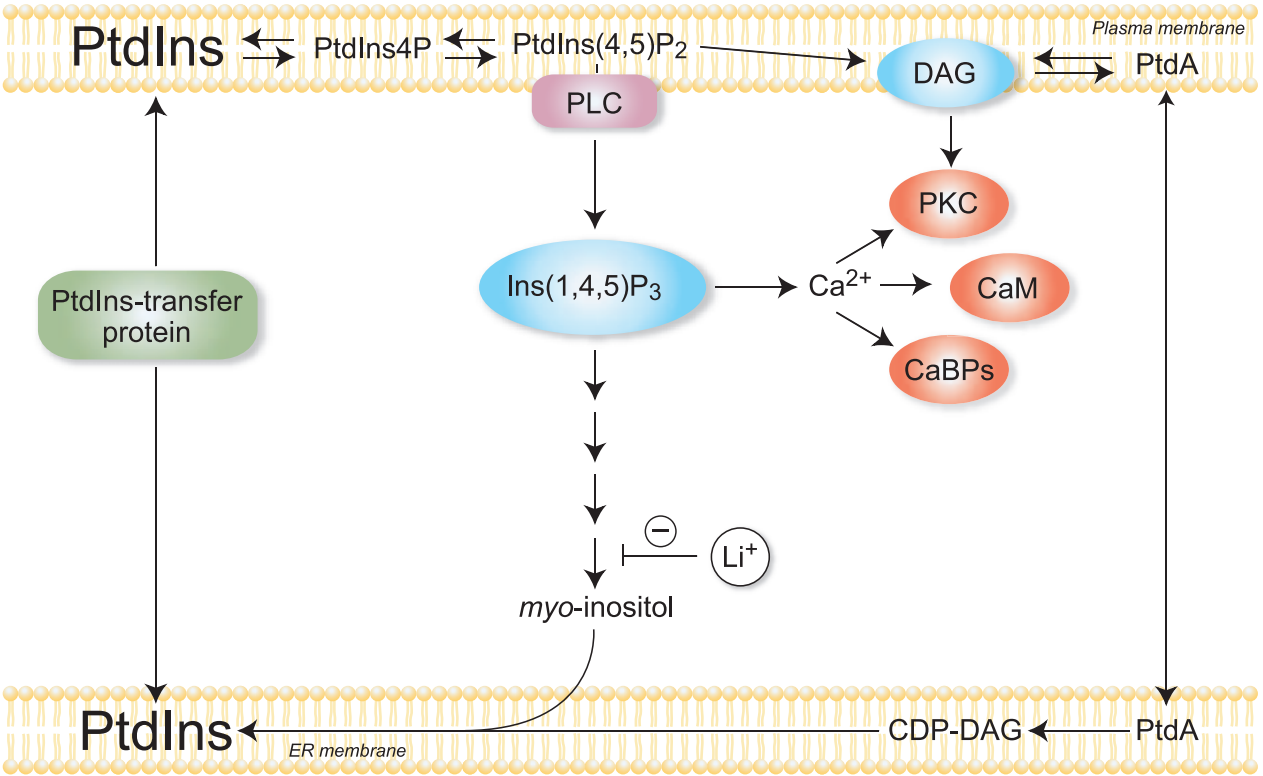

Figure 1 Updated version of the classical 'PI cycle'. Plasma membrane PtdIns(4,5)P_2 is hydrolyzed by PLC enzymes, after receptor stimulation, to generate two second messengers, Ins(1,4,5)P_3 and DAG. Ins(1,4,5)P_3, which mobilizes intracellular Ca^{2+}, is rapidly degraded by sequential dephosphorylations to yield *myo*-inositol. (A fraction of the Ins(1,4,5)P_3 is converted to higher inositol phosphates via pathways that differ between plants, yeast and vertebrates (Shears 2004), but it is not shown in the figure for better clarity.) Some of the dephosphorylating enzymes on the pathway of Ins(1,4,5)P_3 degradation are sensitive to inhibition by Li^+ ions, a finding that led to the idea that the therapeutic effects of Li^+ in bipolar disorder might be related to altered signaling affecting primarily hyperactive receptors (Berridge *et al.* 1989). DAG, the other product of PtdnIns(4,5)P_2 hydrolysis, activates PKC enzymes (and some other effectors, such as ion channels or protein kinase D (PKD) – not shown) before being converted to PtdA by DAG-kinase enzymes. PtdA of the plasma membrane can also activate certain effectors (not shown). In the endoplasmic reticulum (ER), PtdA is converted to CDP-DAG, which is conjugated with *myo*-inositol by PI synthase enzyme(s). When cells are stimulated in the presence of Li^+, CDP-DAG accumulates because of the shortage of *myo*-inositol derived from phosphoinositide hydrolysis. The PtdIns of the ER is transferred to the plasma membrane by PI transfer proteins, but it is less clear whether a similar mechanism transfers PtdA between the membranes. Most downstream pathways in this messenger system are regulated by PKC and other Ca^{2+}-responsive protein kinases or phosphatases.

of the inositol phospholipids. With the identification of new forms of inositol lipids and newly discovered functions of their classical forms, came the cloning of the multiple forms of inositide lipid kinases (Fruman *et al.* 1998) and phosphatases (Majerus *et al.* 1999), several of which have also been linked to human diseases (Pendaries *et al.* 2003) (Fig. 3). Research into the calcium aspect of $InsP_3$-mediated signaling has also shown an enormous expansion, starting with the cloning of the $InsP_3$ receptor (Furuichi *et al.* 1989, Mignery *et al.* 1989) and the continuing quest to identify the molecular entities behind the store-operated Ca^{2+} entry (SOC) pathway (Parekh & Putney 2005). There are several excellent reviews covering almost every aspect of inositide research from the viewpoint of the protein classes as indicated above. This review cannot be a compilation of all of these areas. Instead, it will aim to summarize recent advances and open questions from selected areas of endocrine research pointing to the inositol lipid angle within those areas. This is a selection with an inevitable bias and is by no means an indication that other aspects of inositides are not important in endocrinology. After all, it could be claimed that there are only two kinds of research: one in which phosphoinositides are already implicated and another where their involvement is yet to be recognized!

Receptor-mediated $InsP_3$ formation and Ca^{2+} release

When Ca^{2+}-mobilizing receptors engage their agonist ligands, the subsequent activation of PLC enzymes at the plasma membrane leads to rapid breakdown of

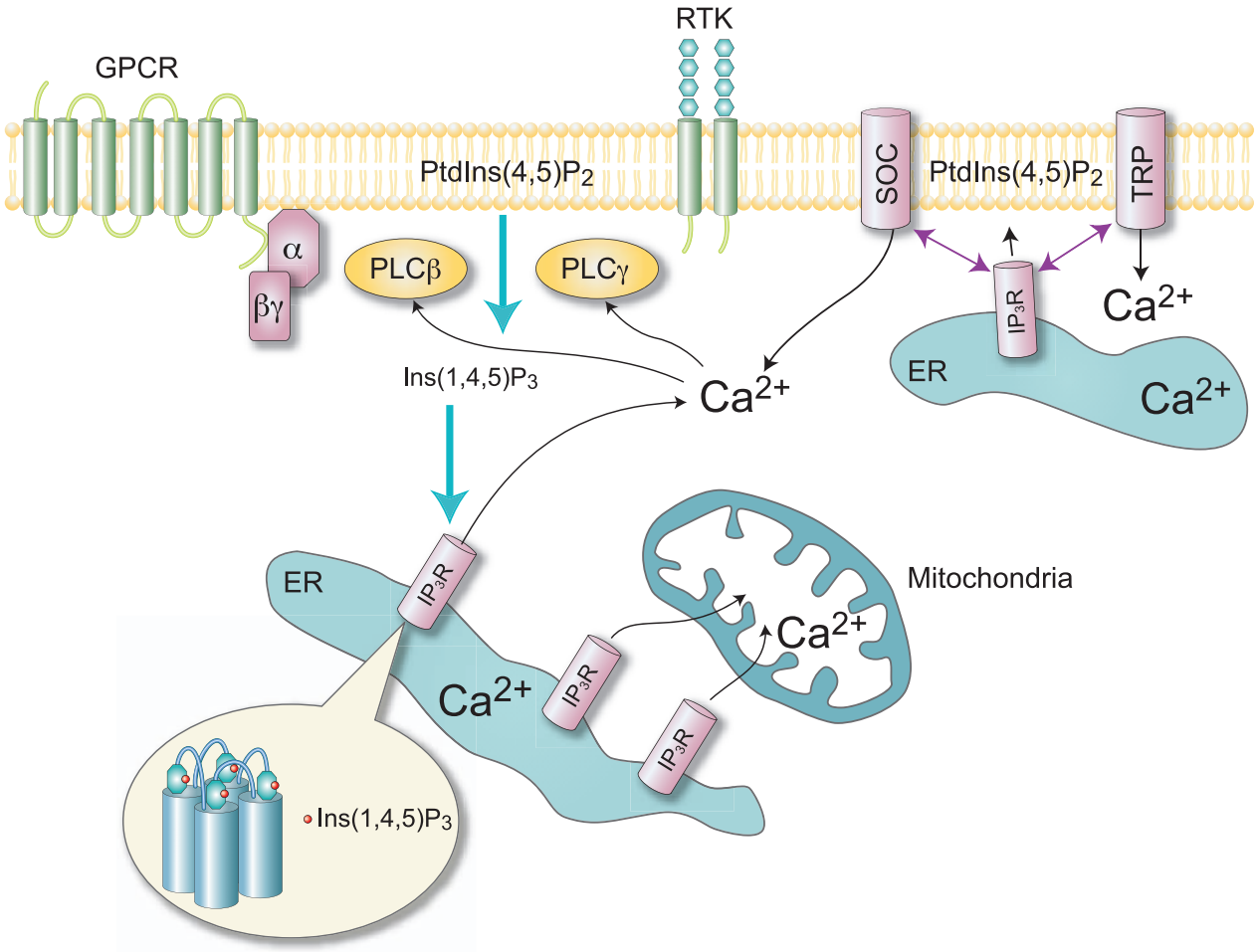

Figure 2 G protein-coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs) activate PtdIns(4,5)P_2 hydrolysis by distinct mechanisms, the former using G protein-mediated activation of PLCβ isoforms, and the latter causing PLCγ activation. Ins(1,4,5)P_3 binds to its receptors located primarily in the ER in the form of tetrameric Ca^{2+} channels releasing Ca^{2+} from the internal stores. The N-terminal Ins(1,4,5)P_3-binding domain of the InsP_3 receptor is probably in close proximity to the C-terminal channel domain with a long intervening regulatory domain (see insert). Emptying of the ER Ca^{2+} pools relays information (by unknown mechanisms) to plasma membrane Ca^{2+} channels, commonly termed store-operated Ca^{2+} channels (SOC), to enhance Ca^{2+} influx into the cell. Members of the TRP family of Ca^{2+} channels may function as SOC, and it has been suggested that there is a physical regulatory contact between plasma membrane-adjacent InsP_3 receptors and certain TRP channels (see text for details). Another spatial organization is related to the 'coupling' of Ca^{2+} release via the InsP_3 receptors with uptake by mitochondria that are juxtaposed to InsP_3 receptor-rich ER domains.

PtdIns(4,5)P_2. This process is both amplified and at the same time self-limited in a number of ways. It is amplified because of the positive regulation of PLC enzymes by cytoplasmic Ca^{2+}, an effect that accounts for the profound impact of Ca^{2+} release on the extent of PtdIns(4,5)P_2 hydrolysis (e.g. Horowitz *et al.* 2005). Without receptor activation, Ca^{2+} per se is much less potent, and only large cytoplasmic Ca^{2+} increases lead to PtdIns(4,5)P_2 breakdown. Although all PLC enzymes are Ca^{2+}-sensitive, the PLCδ1 enzyme appears to be largely responsible for Ca^{2+}-mediated positive feedback on InsP_3 generation (Rhee 2001). At the same time, the process of InsP_3 formation is limited because most GPCRs undergo homologous desensitization that limits their ability to activate G-proteins (Luttrell & Lefkowitz 2002). Moreover, both GPCRs and RTKs undergo ligand-induced endocytosis and are rapidly removed from the plasma membrane, whence they can also be quickly recycled and reappear during resensitization (Gaborik & Hunyady 2004). PtdIns(4,5)P_2 hydrolysis is also limited because, as a result of its relatively low level in the membrane, it has to be replenished by the sequential actions of PI 4- and PIP 5-kinases on the larger pools of PtdIns. Even the larger pool of plasma membrane PtdIns can become depleted if not maintained by supply from the ER by PI transfer proteins (Fig. 1).

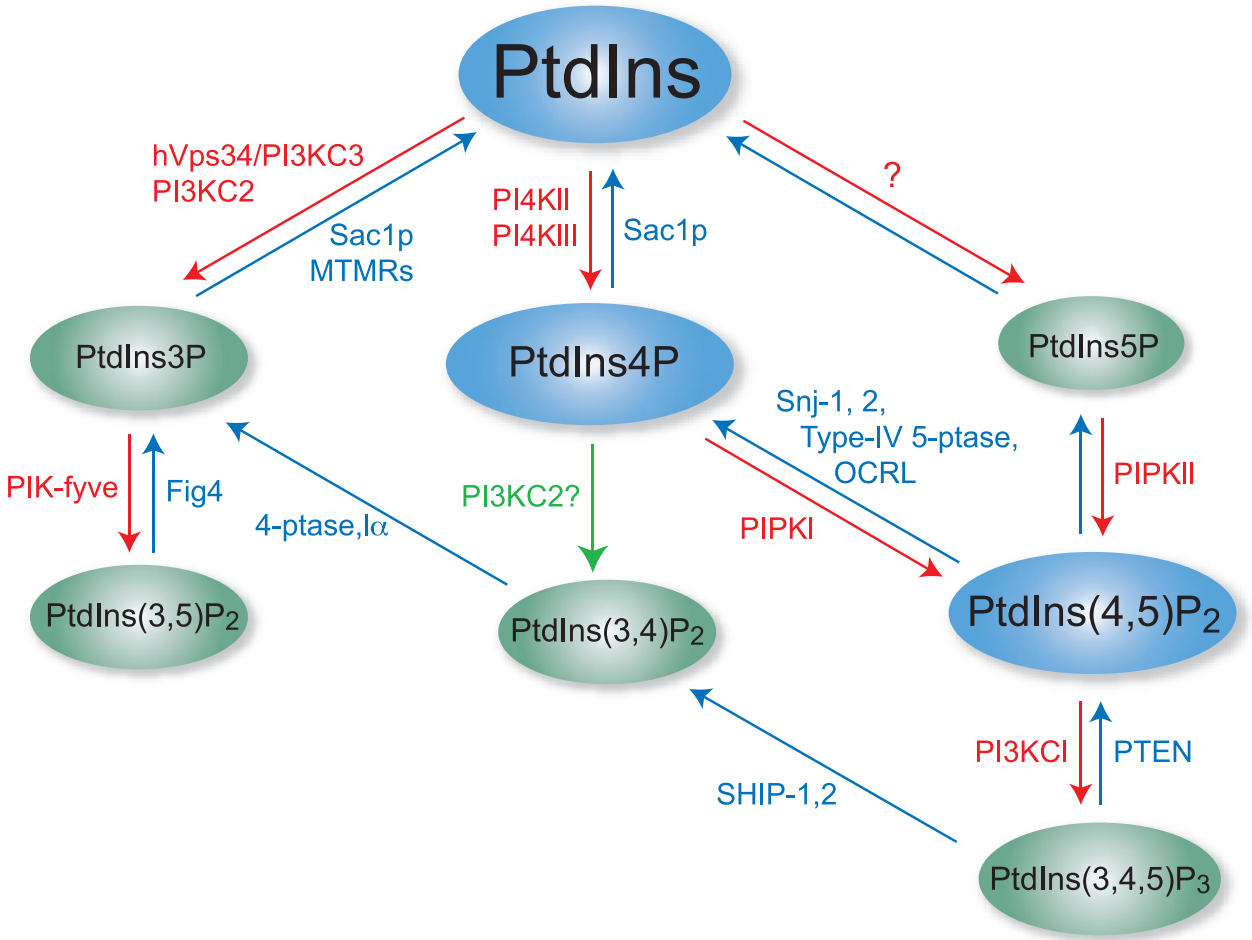

Figure 3 The network of interconversions between phosphoinositides. Phosphoinositides are phosphorylated (red arrows) by inositide kinases and are dephosphorylated (blue arrows) by phosphoinositide phosphatases. Most (but not all) of the kinases are quite specific and phosphorylate only one substrate in a specific position on the inositol ring. Some kinases (such as the class II PI 3-kinases, PI3KC2) show wider substrate tolerance *in vitro* but are likely to have a clear substrate preference *in vivo*. The substrate specificity of most (but not all) phosphatases is less stringent *in vitro*, and some can dephosphorylate lipids as well as the soluble inositol phosphates. The physiologic and preferred substrate is not known for all inositide phosphatases, nor is it known in all cases which enzyme or enzymes are primarily responsible for a specific interconversion step. For example, Sac1 phosphatases can remove the 3- and 4-phosphates from a mono-phosphorylated inositol lipid, but synaptojanins (Snj), which are primarily known as 5-phosphatases, also contain a Sac1 phosphatase motif and can function as Sac1 phosphatases (Guo *et al.* 1999). Since myotubularin-related inositol lipid phosphatases (MTMRs) (Taylor & Dixon 2003) also dephosphorylate the 3 position on PtdIns(3)P, it is likely that PtdIns(3)P is dephosphorylated by Sac1, Snj-s and MTMRs in different cellular compartments (Parrish *et al.* 2004). This figure indicates only the more established routes; for all other possibilities of interconversion, see Abel *et al.* (2001).

Which inositol lipid pools participate in the classical signaling cascade and which enzymes are primarily responsible for their generation?

The existence of hormone-sensitive and insensitive pools of phosphoinositides has been described in early research using metabolic labeling with myo-inositol (Koreh & Monaco 1986). Recent studies have indicated that PtdIns(4,5)P_2 is produced by two different pathways: in the canonical pathway, it is formed through PtdIns(4)P by sequential phosphorylations by PI 4-kinases (Balla 1998) and the type I PIP kinases (or PIP5Ks) (Hinchliffe *et al.* 1998). In a recently recognized pathway, PtdIns(4,5)P_2 synthesis proceeds via PtdIns(5)P that is phosphorylated by type-II PIP kinases (or PIP4Ks) (Rameh *et al.* 1997) (Fig. 3). Since each group of enzymes has multiple forms that are located in distinct cellular compartments, it is inevitable that phosphoinositides will be generated in different types of membranes. Which of these inositide pools are linked to hormone action? It is widely assumed that hormonal stimulation leads to breakdown of the plasma membrane PtdIns(4,5)P_2 pool, and this has been

substantiated by the use of green fluorescent protein (GFP)-fused pleckstrin homology (PH) domains that recognize the lipid in the plasma membrane. However, PtdIns(4,5)P_2 pools also exist in other membrane locations, such as the Golgi, the ER and the nucleus (Watt *et al.* 2002), and PLC enzymes are also found at these sites (Rebecchi & Pentyala 2000). Therefore, it is not unreasonable to assume that PLC activation – either direct, or mediated by the cytoplasmic Ca^{2+} increase – occurs at intracellular membranes and contributes to $InsP_3$ and DAG generation at those sites with local consequences.

Equally important is the simple question of which enzymes generate the PI(4,5)P_2 that is subject to receptor-controlled PLC-mediated hydrolysis. Recently, it was shown that human PIP 5-kinase γ is the enzyme that is necessary for GPCR-mediated $InsP_3$ formation and Ca^{2+} signaling (Wang *et al.* 2004). In contrast, mouse PIP 5-kinase Iβ (identical to human PIP5KIα), recruited by Bruton's tyrosine kinase, enhances Ca^{2+} signaling in B cells (Saito *et al.* 2003). Stimulation of PIP 5-kinase activity by small guanosine triphosphate (GTP)-binding proteins has been well documented (Chong *et al.* 1994, Honda *et al.* 1999), but it is not known whether receptor-mediated activation of PIP 5-kinase activity occurs. A very rapid increase in PtdIns(4,5)P_2 level after stimulation has been recently demonstrated, suggesting direct activation of PtdIns(4,5)P_2 synthesis (Xu *et al.* 2003). Less clear is the identity of the PI 4-kinase or kinases that provide PtdIns(4)P for the PIPKs. Hormone-sensitive PtdIns(4)P pools have been shown to be generated by the type-III PI4Ks (which are sensitive to higher concentrations of PI3K inhibitors) (Nakanishi *et al.* 1995). However, neither the PI4 KIIIα nor -β isoform is detectable at the plasma membrane of mammalian cells; instead, these isoforms are located in ER and Golgi membranes respectively (Wong *et al.* 1997). Yet, recent studies have shown that a plasma membrane (PM) pool of PtdIns(4)P is generated by PI4 KIIIα (Balla *et al.* 2005), raising the question of whether the lipid is generated at the PM by a small fraction of the enzyme or is generated elsewhere and transported to the PM. There is little direct evidence that PI4K activities are stimulated during receptor activation, and only PI4 KIIβ has been shown to be activated by a Rac-dependent mechanism (Wei *et al.* 2002).

As mentioned above, the maintenance of PtdIns(4,5)P_2 pools also requires the function(s) of the family of PI transfer proteins (PITPs). These proteins transfer PtdIns and PtdCho between membranes and are necessary for maintenance of the PLC-sensitive phosphoinositide pools (Thomas *et al.* 1993). PITPs also exist in several forms, of which the soluble PITPα and PITPβ isoforms have non-overlapping functions that reach far beyond the regulation of hormone-sensitive phosphoinositide pools, affecting both phospholipid synthesis and vesicular trafficking. This function of lipid-transfer proteins will be further detailed below.

How does $InsP_3$ regulate Ca^{2+} release?

Since the isolation and cloning of the $InsP_3$ receptor channels, impressive progress has been made in understanding their functions. All three isoforms of the $InsP_3$ receptor (types I, II and III) function as intracellular Ca^{2+} channels that operate as a homo- or heterotetramer (Mikoshiba 1993). Each receptor subunit has a channel portion containing six transmembrane helices and a pore domain located between TM5 and TM6, close to the C-terminus of the protein (Maeda *et al.* 1991, Galvan *et al.* 1999, Patel *et al.* 1999). The ligand-binding domain (LBD) of the receptor is located at the N-terminus (Mignery & Sudhof 1990) and is separated from the channel domain by a long intervening regulatory region facing the cytoplasm (Mignery & Sudhof 1990, Mikoshiba 1993). $InsP_3$ binding leads to rapid activation of the channel, but Ca^{2+}-induced Ca^{2+} release, similar to that featured in the related ryanodine receptors (RyR), is also an important regulatory mechanism of IP_3Rs (Taylor & Laude 2002). Because of their ER location, little is known about the gating mechanism and properties of IP3R channels. The only electrophysiologic data are derived from isolated channels reconstituted in lipid bilayers (Bezprozvanny *et al.* 1991) or from patch-clamp recordings of the nuclear envelope (Mak & Foskett 1994). Most of our knowledge of the behavior of the intact channels is inferred from measurements of Ca^{2+} (or other cation) fluxes. These studies have provided invaluable information about the $InsP_3$ and Ca^{2+} regulation of the channel (Bezprozvanny *et al.* 1991, Hajnoczky & Thomas 1994), but no molecular mechanisms or states have been correlated with channel behavior. One major question is how the N-terminal LBD can regulate the channel itself. Recent evidence suggests that the C-terminal channel domain and the N-terminal LBD are in very close molecular proximity, and that the ligand-induced conformational change within the LBD could be transferred to the channel domain itself (Boehning & Joseph 2000). The recently solved X-ray structure of the LBD (Bosanac *et al.* 2002) and the adjacent N-terminal inhibitory domain (Bosanac *et al.* 2005) has helped to clarify the structural basis of $InsP_3$ binding, and, together with high-resolution electron microscopy and 3-D reconstruction of the channel structure (Jiang *et al.* 2002, da Fonseca *et al.* 2003, Sato *et al.* 2004), should advance our understanding of the gating mechanism of the protein.

The positioning of ER membranes containing $InsP_3$ receptors relative to other membranes, and the interaction of the receptor with other proteins, add to the complexity of Ca^{2+} regulation in local compartments (Fig. 2). Early fractionation studies suggested that $InsP_3$ receptors could be isolated from 'mitochondrial' (Dawson & Irvine 1984) and 'plasma membrane' (Guillemette *et al.* 1988) fractions, indicating ER contamination of these fractions enriched in $InsP_3$ receptors. Recent evidence has shown the

existence of a special ER–mitochondria interface (Rizzuto *et al.* 1993) and a very close 'quasi-synaptic' functional coupling between $InsP_3$ receptor-mediated Ca^{2+} release and mitochondrial Ca^{2+} uptake (Csordas *et al.* 1999). Moreover, the physical association of $InsP_3$ receptors with TRPC3 channels at the plasma membrane could provide the basis for Ca^{2+} influx regulation linked to $InsP_3$-induced Ca^{2+} release (Kiselyov *et al.* 1998). These observations raise the question of whether the $InsP_3R$ could serve as a structural participant in the tethering of the ER to other membranes in the proximity of the channel. It has also been suggested that the LBD of the $InsP_3R$ binds to $PtdIns(4,5)P_2$ of the plasma membrane in quiescent cells when $InsP_3$ levels are low (Glouchankova *et al.* 2000), and that changing $PtdIn(4,5)P_2$ together with increased $InsP_3$ could also participate in the regulation of these channels.

Cell-surface receptors and PI3K-mediated signaling

The classical activation mechanism of PI3Ks was identified by studies on receptor tyrosine kinases and on soluble and oncogenic tyrosine kinases (Otsu *et al.* 1991). In these systems, the p85 regulatory subunit associates with tyrosine-phosphorylated target sequences through its SH2 domains, recruiting the cytoplasmic PI3Kα or -β catalytic subunit to the membrane. The p85 subunit becomes tyrosine phosphorylated during this process, leading to increased activity of the kinase. Less is known about the mechanism of PI3K activation in the case of GPCRs. In hematopoietic cells, where PI3Kγ is found in significant amounts, activation via the βγ-subunits of Gi/Go proteins is the main activation pathway (Stephens *et al.* 1994). This is mediated by associated p101 (Stephens *et al.* 1997) or p84 (Suire *et al.* 2005) regulatory subunits, but direct regulation of the PI3Kγ enzyme by βγ-subunits has also been reported (Leopoldt *et al.* 1998). Much less clear and more controversial is the manner in which PI3K is activated by GPCRs in tissues where PI3Kγ is not expressed, or is present only at low levels. In many cases, activation occurs by transactivation of receptor tyrosine kinases (Daub *et al.* 1996) followed by the above-detailed mechanism, but this is not the sole means by which GPCRs activate PI3Ks. It is also not known what determines which of the class I PI3Ks (α,β,δ) is activated and which splice form of the p85/p55 regulatory subunit associates with them. Deletion of either PI3Kα or β is lethal (Bi *et al.* 1999, 2002), as is the elimination of all the splice forms of the p85/55α subunits (Fruman *et al.* 2000), but not of the p85α form alone (Terauchi *et al.* 1999), indicating a level of complexity that is still far from being understood. Impairment of PI3K signaling has prominent effects on insulin signaling (Terauchi *et al.* 1999), and recent studies indicate that the stochiometry between the p85 and p110 subunits, together with the direct interaction of the free p85 subunit with IRS-1, is a major factor determining the insulin responsiveness of the cells (Luo *et al.* 2005).

Activation of PI3K by estrogens via ligand-dependent association of ERα with the p85 regulatory subunit has been demonstrated (Simoncini *et al.* 2000). More recently, a heptahelical receptor, GPR30, which is located in the ER, was shown to respond to estrogen stimulation, leading to the activation of PI3K in the nucleus, as indicated by the translocation of the Akt PH-domain to the nucleus (Revankar *et al.* 2005). Many questions remain to be answered about this intriguing effect, but it appears to be a new paradigm in steroid hormone action that is not mediated by the classical nuclear receptors.

The main downstream signaling pathway from PI3Ks proceeds via the Akt protein kinase (Franke *et al.* 1997) and its upstream regulator kinase, PDK1 (Alessi *et al.* 1997). These kinases are master regulators of a whole range of cellular processes related to glucose metabolism, protein synthesis and cell division, and also represent the main antiapoptotic pathway. Detailed coverage of these processes in endocrine and metabolic regulation is beyond the scope of this review, but can be found elsewhere (Mora *et al.* 2004).

Receptor trafficking and its regulation by phosphoinositides

Many cell-surface receptors undergo ligand-induced endocytosis mostly (but not exclusively) by a clathrin-mediated internalization process that shares many of the characteristics of the endocytosis and recycling of nutrient receptors (Brown & Goldstein 1979). The sorting of the receptors into clathrin-coated pits is mediated by interaction of the receptor with clathrin-adapter proteins. Several adapter proteins have been identified, including the tetrameric adapters, AP-(2–4), the monomeric adapters such as AP-180 (or its nonneural form, CALM), the Dab1/ARH and the GGA proteins. Many of these adapters exert their effects at intracellular membranes (Owen *et al.* 2004), when they recognize specific sequences (sorting motifs) within the intracellular segments of nutrient receptors or RTKs. They also bind to clathrin, thereby bringing the receptors to the site of clathrin assembly (Fig. 4). Several sorting motifs have been identified in receptors, some containing Tyr, such as the NPxY or the YxxΦ sequences, and others containing Leu/Ile residues, such as the D/ExxxL/I or DxxLL motifs (Bonifacino & Traub 2003). In the case of GPCRs, the common adapter proteins are β-arrestins which bind to GPCR tails that are Ser/Thr phosphorylated by G protein receptor kinases (GRKs) (Lefkowitz 1993). Phosphorylation of GPCR tails by GRKs is greatly facilitated by the binding of agonist ligands, and it accounts for the ligand-dependence of GPCR internalization. The role of receptor internalization is obvious in the case of nutrient

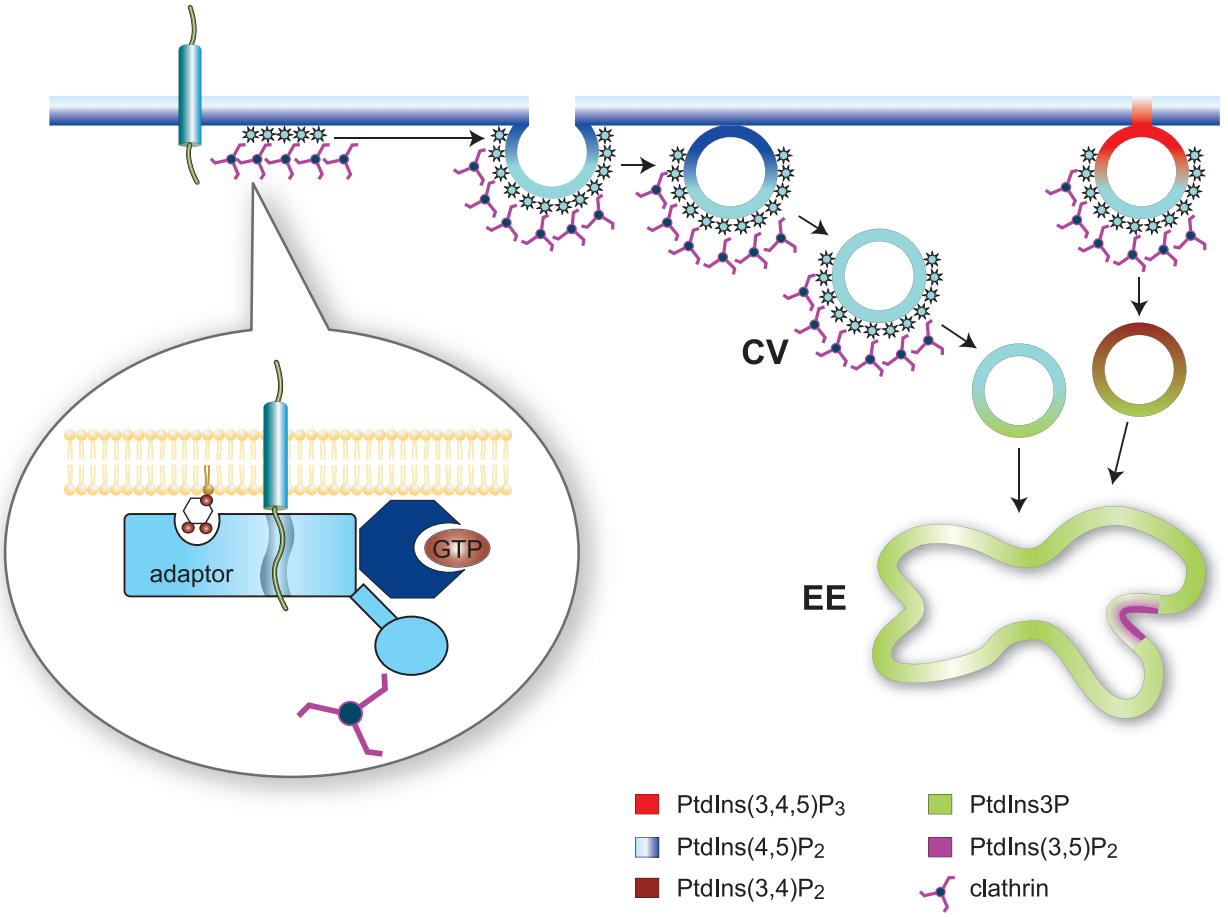

Figure 4 The general scheme of endocytosis from the plasma membrane. Transmembrane proteins (e.g. receptors) associate with adapter proteins capable of recognizing the internalization signal (usually within their membrane-adjacent cytoplasmic sequence). (Often, a covalent modification, such as phosphorylation or ubiquitination, takes place at these sites on the transmembrane proteins – not shown here.) The adapter protein interaction is also aided by the presence of membrane phosphoinositides and requires an active small GTP-binding protein (such as Arfs or Rabs). The adapter protein also binds clathrin, thereby recruiting the protein into clathrin-coated pits. Several adapter proteins have been shown to bind phosphoinositides, which, in most cases, are the most abundant PtdIns(4,5)P_2. Once the vesicles are pinched off (clathrin-coated vesicles, CV), this lipid is no longer demonstrable in the vesicles and is replaced by 3-phosphorylated inositides, contributing to a new identity of the vesicles. Recent studies also indicate that PtdIns(3,4,5)P_3 formed in the membrane can be a source of 3-phosphorylated lipids in the endosomes after sequential dephosphorylations by vesicle-associated phosphatases that remove the 5- and 4- phosphates (Ivetac *et al.* 2005, Shin *et al.* 2005). Multivesicular bodies are formed from early endosomes (EE) after inward invagination and budding, and PtdIns(3,5)P_2 is a critical component of this process (see text for more details).

receptors but is less clear in the case of hormone receptors. For this latter, it is an important mechanism for regulating the number of available receptors on the cell surface, and it also eliminates activated receptors that often undergo degradation (Dikic 2003). There is also increasing evidence that some internalized receptors may continue to signal in the endocytic compartments (Luttrell & Lefkowitz 2002).

It is quite remarkable that many of the proteins that participate in the internalization process contain binding sites for phosphoinositides. With the exception of the GGAs, all of the above-mentioned clathrin-adapter proteins have been shown to contain one or more phosphoinositide-binding motifs (Owen *et al.* 2004). Some GRKs (such as GRK2 and 3) contain a pleckstrin-homology domain that is important for the membrane recruitment of the enzymes (Pitcher *et al.* 1998), but the phospholipid species involved in their regulation is not yet known (Carman *et al.* 2000). Recently, β-arrestins were shown to contain phosphoinositide-binding regions that are important for GPCR internalization (Gaidarov *et al.* 1999, Lee *et al.* 2003). Finally, the GTP-binding protein, dynamin, which is critical for pinching off internalized vesicles from the plasma membrane, is also known to contain a PH domain. In this case, mutation of the residues essential for lipid binding has a dominant negative

effect on receptor endocytosis (e.g. Lee *et al.* 1999). From the presence of these domains in the above proteins and their ability to bind phosphoinositides *in vitro*, it is widely assumed that phosphoinositides regulate receptor endocytosis. Mutagenesis studies targeting the lipid-binding regions of the proteins support this assumption, but few studies are available on the question of whether modifying lipid production would affect receptor trafficking. Wortmannin-sensitive (i.e., type III PI4K-synthesized) PtdIns(4)P and PtdIns(4,5)P_2 pools support muscarinic and β2-adrenergic receptor endocytosis (Sorensen *et al.* 1998), and the plasma membrane recruitment of AP-2 protein and transferrin receptor endocytosis correlates with PIP5KIβ activity (Padron *et al.* 2003). In general, it is not certain which phosphoinositide species regulates any particular step in the complex process of endocytosis. Because of its abundance and *in vitro* binding to many of the above domains, PtdIns(4,5)P_2 is considered the most important inositide in receptor endocytosis. Although several reports have indicated that PI 3-kinases are also agents in this process (Joly *et al.* 1994, Naga Prasad *et al.* 2002), pharmacologic blockade of PI3Ks does not dramatically influence the early steps of receptor internalization (Hunyady *et al.* 2002). Most recently, the protein kinase activity of PI3Kγ via phosphorylation of tropomyosin was shown to be critical for β-adrenergic receptor endocytosis (Naga Prasad *et al.* 2005).

Fate of the internalized receptors

It is also well documented that the subsequent fate of internalized receptors is highly dependent on processes regulated by PI3Ks. After reaching early endosomes, a large proportion of receptors recycle back to the plasma membrane via a vesicular mechanism involving Rab4 and Rab5 GTPases (Seachrist *et al.* 2000, Hunyady *et al.* 2002, Dale *et al.* 2004). This rapid recycling process is inhibited by the PI 3-kinase inhibitors, wortmannin and LY294002, leading to the accumulation of the receptors in large endocytic vesicles (Shpetner *et al.* 1996, Hunyady *et al.* 2002). A fraction of the transferrin receptors and some GPCRs are also sorted into another compartment, called recycling endosomes, that is positive for Rab11 GTPase, and from which there is a significantly slower recycling to the cell surface by a process more resistant to PI 3-kinase inhibitors (Hunyady *et al.* 2002, van Dam *et al.* 2002). Interestingly, a group of GPCRs very rapidly recycle back to the cell surface after internalization, and these receptors are not sorted into, and do not take β-arrestin, to 'deeper' compartments (Zhang *et al.* 1999). One of the most exciting 'organelles' of the internalization and recycling pathway is the multivesicular body (MVB), a site for molecular decisions on whether receptors recycle or undergo degradation. Ubiquitination of receptors often determines their fate. Mono-ubiquitination (sometimes at multiple sites) has been shown to be important for internalization and targeting of activated RTKs, such as the EGFR to the inner membrane of the MVB and subsequent lysosomal degradation (Hicke 2001, Katzmann *et al.* 2002, Haglund *et al.* 2003). This process is to be distinguished from poly-ubiquitination (where the added ubiquitin is further ubiquitinated multiple times) of soluble proteins that are targeted for proteasomal degradation (Bonifacino & Weissman 1998), although the distinction between the two kinds of ubiquitination is not always clear. Unlike transferrin receptors, some GPCRs also appear at the MVB during their recycling or degradation (Hunyady *et al.* 2002), and also have been shown to be ubiquitinated (Marchese & Benovic 2001, Shenoy *et al.* 2001). The role and significance of GPCR ubiquitination in the endocytic and recycling process is not as well understood as for RTKs. 3-Phosphorylated inositides are principal regulators of the sorting process along the endocytic pathway. Class III PI3Ks generate PtdIns(3)P, which contributes to the recruitment to endocytic vesicles of proteins containing FYVE, or PX domains such as Hrs or sorting nexins, respectively. These proteins are important in cargo selection and vesicle dynamics. PtdIns(3)P is also converted to PtdIns(3,5)P_2 by the PIKfyve enzyme (Shisheva *et al.* 1999) (also termed type III PIP kinase). In *Saccharomyces cerevisiae*, this kinase, termed Fab1p, is also needed for protein sorting into the MVB (Odorozzi *et al.* 1998).

These selected examples illustrate the complexity of the phosphoinositide requirement of the endocytic and sorting process, and highlight the magnitude of the task that remains to be completed in clarifying the molecular details and biologic importance of receptor endocytosis in endocrine functions.

Phosphoinositides and ion channels

The relationship between phosphoinositide turnover and Ca^{2+} signaling has long been firmly established, and for a while the only debate was about which was the consequence of the other (Cockcroft 1981, Michell 1982). However, the discovery of $InsP_3$-mediated Ca^{2+} release from internal pools has clarified the primary connection between them. Importantly, Ca^{2+} also enters the cells via Ca^{2+} channels located in the plasma membrane, and initially a distinction was made between voltage-gated Ca^{2+} channels, which mostly regulate the functions of 'excitable' cells, and non-voltage-gated Ca^{2+} influx pathways (or receptor-regulated Ca^{2+} channels) working in nonexcitable and endocrine cells. Although this distinction has not persisted very long (e.g., the presence of voltage-gated Ca^{2+} channels in endocrine cells has become common knowledge), the molecular entities behind the Ca^{2+} entry mechanism(s), described as 'capacitative' Ca^{2+} entry pathway or store-operated Ca^{2+} entry (SOC) (Putney 1986), has remained elusive.

All nonexcitable cells display increased Ca^{2+} influx that falls within the criteria of capacitative or SOC, after activation by a 'calcium-mobilizing' stimulus (Putney 1986). After decades of studies to identify the Ca^{2+} channels that underlie this phenomenon, it is still questionable whether a single molecular entity is responsible for the enhanced Ca^{2+} influx observed after emptying of the intracellular Ca^{2+} stores (Parekh & Putney 2005). The current activated by the release of Ca^{2+} stores is termed I_{CRAC} (calcium release-activated current) (Hoth & Penner 1992), but its molecular equivalent is still unresolved, as is the question of whether I_{CRAC} is 'the current' corresponding to SOC (see Parekh & Putney (2005) for an excellent recent review). The Ca^{2+} channel underlying the *Drosophila* transient receptor potential (Trp) in the fly photoreceptor was proposed to be a channel corresponding to SOC (Wes *et al.* 1995). After cloning of the mammalian homologs of these proteins ($TrpC_{1–7}$, 'C' stands for classical), a large number of studies addressed this question. Of the seven TrpC channels, most data were gathered for TrpC1 and TrpC3 channels. Overexpression of TrpC1 channels yields a nonselective Ca^{2+}-permeable conductance that moderately increases Ca^{2+} influx after store depletion in some studies (Zhu *et al.* 1996), but not in others (Lintschinger *et al.* 2000). This discrepancy could be due to the fact that TrpC1 proteins require coexpression of other Trp channels (such as TrpC4 and -5) to traffic properly to the plasma membrane (Strubing *et al.* 2001). Downregulation or elimination of TrpC1 channels at the cellular level also indicates that they are at least partially responsible for store-operated Ca^{2+} influx (Liu *et al.* 2003) and I_{CRAC} (Mori *et al.* 2002).

Studies on TrpC3 channels provided additional insights into the connection between Ca^{2+} release and influx. Early studies suggested that these channels are regulated by Ca^{2+} and diacylglycerol (and thereby responded to agonist stimulation), but not by store depletion (Lintschinger *et al.* 2000). However, other studies have shown that TrpC3 channels do respond to store depletion and that they physically interact with $InsP_3$ receptors, providing experimental support for the previously hypothetical conformational-coupling model of SOC (Kiselyov *et al.* 1998). It is very likely that Trp channel behavior depends on the presence of other Trp channels (or of associated proteins) in a particular cell, and that cells display a great variety of Ca^{2+} influx characteristics, depending on the composition of the molecular complex responsible for the store-operated influx phenomenon in specific cell types. This may explain why overexpression studies with the various Trp channels yield apparently discordant information. A detailed, up-to-date review of all Trp channels is given by Putney (2004). Very recent data identify STIM1, an ER-resident, single-transmembrane protein with a Ca^{2+}-sensing luminal domain, as a critical component of SOC (Liou *et al.* 2005, Roos *et al.* 2005). STIM1 is not a channel itself, but it may finally provide us with a thread from which the SOC phenomenon will be deciphered. There is very little information about direct regulation of TrpC channels by phosphoinositides, but findings with members of other Trp channel families (see below) indicate that this question should be further investigated.

Curiously, the first ion channels for which phosphoinositide regulation was described were not Ca^{2+} channels, but members of the inwardly rectifying (Kir) potassium channel family (Hilgemann & Ball 1996). Some Kir channels were shown to be regulated by the βγ-subunits of heterotrimeric G proteins (GIRKs) (Krapivinsky *et al.* 1995) and $PtdIns(4,5)P_2$ (Huang *et al.* 1998, Zhang *et al.* 1999) and became the prototypical examples. A number of studies followed that showed phosphoinositide regulation of other ion channels. These included other potassium channels, such as the M-current (Suh & Hille 2002) and the underlying KCNQ channels (Zhang *et al.* 2003), and the two-pore domain K^+ channels (Chemin *et al.* 2005, Lopes *et al.* 2005). Among Ca^{2+} channels, members of the Trp family (other than TrpCs) have been shown to be regulated by $PtdIns(4,5)P_2$. As shown for the TrpM5, -7 and -8 channels, $PtdIns(4,5)P_2$ is necessary for channel activity (Runnels *et al.* 2002). In the case of TrpM8, both cooling and addition of activator ligands, such as menthol, alter the lipid affinity of the channel. The region responsible for lipid regulation has been mapped within the so-called Trp-domain (Rohacs *et al.* 2005). Other Trp channels, such as the vanilloid receptor (TrpV1), are also regulated by $PtdIns(4,5)P_2$ (Chuang *et al.* 2001), and this lipid was found to be essential for the recovery of the TrpV1 channel from desensitization (Liu *et al.* 2005). The intimate relationship between some of these channel proteins and PLC enzymes strongly suggests that the Ca^{2+} that enters via Trp channels controls local $PtdIns(4,5)P_2$ levels, thereby regulating channel activity by a local feedback regulation. Whether this local control is a general principle governing the function of other Trp channels, including the 'classical' TrpC channels discussed above, remains to be determined.

A further emerging theme in Ca^{2+} (and other) channel control is that channels located in intracellular membrane compartments are inserted into the plasma membrane by a regulated process quite reminiscent of the GLUT4 glucose transporter insertion into the plasma membrane after insulin stimulation. It has been recently shown that growth factors enhance the insertion of TrpC5 channels from a vesicular pool by a PI3K- and Rac1-mediated mechanism that also involves PIP5KIα (Bezzerides *et al.* 2004). Similarly, PI3K-dependent trafficking of voltage-gated Ca^{2+} channels to the plasma membrane has been recently reported (Viard *et al.* 2004). Intriguingly, a previously described curious stimulatory effect of PLCγ1 expression on the activity of TrpC3 channels (that oddly did not require the PLC activity of the protein) has also

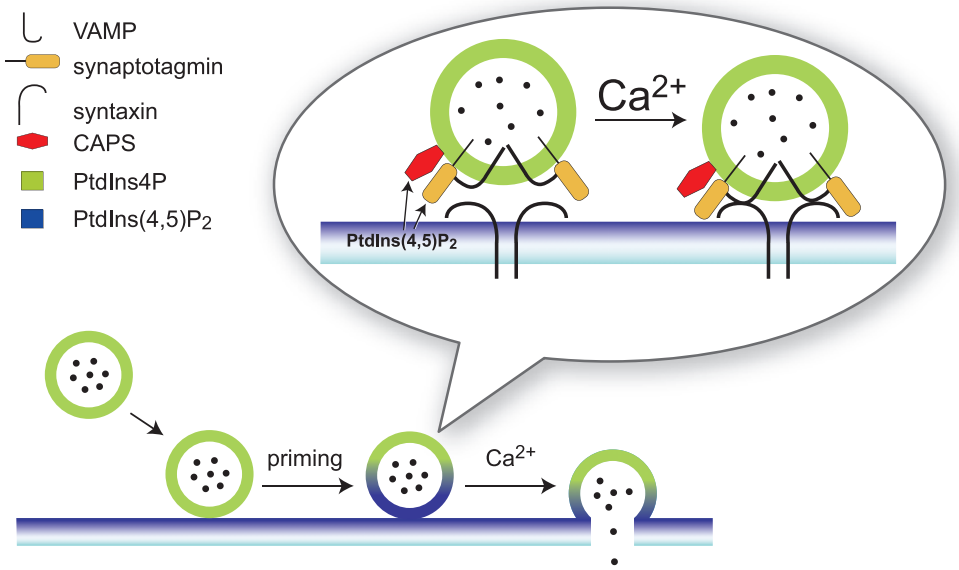

Figure 5 Multiple steps in the exocytic process. Secretory vesicles containing their cargo become docked to the plasma membrane to form the rapidly releasable vesicular pool by maturation. During maturation, which is an ATP-dependent process, these vesicles become 'primed', that is, ready for release once the Ca^{2+}-induced triggering takes place. Synthesis of phosphoinositides (probably PtdIns(4,5)P_2) is part of the priming process, and the recruitment of the Ca^{2+}-binding regulatory protein CAPS (which also binds PtdIns(4,5)P_2) is an essential component of priming. Vesicles are stabilized by the t- and v-snare proteins (syntaxin and VAMP respectively), and they respond to Ca^{2+} increases via synaptotagmin (a protein also capable of phosphoinositide binding), which ultimately triggers the conformational change(s) leading to pore opening and cargo release. Phosphoinositides as well as Ca^{2+} increases act at multiple steps, and production of the lipid both at the membrane and on the surface of the vesicles may take place.

been attributed to enhanced surface expression of the channels (Patterson *et al.* 2002). In this newly discovered process, one of the 1/2 PH-domains of PLCγ1 and a half-PH domain located within the intracellular tails of TrpC channels form an 'intermolecular PH domain' providing phosphoinositide interaction and stabilizing the channel at the plasma membrane (van Rossum *et al.* 2005). These new observations demonstrate that, in addition to the acute regulation of channel activities in the plasma membrane by rapid changes in phosphoinositides, these lipids also control the trafficking and distribution of the channels between the various membranes, adding a new level of complexity to the control of Ca^{2+} (and other ion) fluxes of the cell.

Phosphoinositides and exocytosis

While regulated secretion is one of the key features of many endocrine and neuroendocrine cells, it is also found in other cells such as those of the immune system. This process is also called dense-core vesicle (DCV) exocytosis to distinguish it from the small synaptic vesicle (SV) exocytosis found in presynaptic terminals, although the underlying processes have many common features (Martin 2003, Wenk & De Camilli 2004). DCVs containing cargo to be secreted undergo maturation that increases their competence to dock, and ultimately fuse, with the plasma membrane when a rapid rise in Ca^{2+} concentration triggers the fusion process. Some of the mature granules under the membrane will dock to the plasma membrane, but these predocked vesicles still have to undergo 'priming' to become the 'readily releasable pool' that is first to be fused upon stimulation (Fig. 5). The prefusion process of 'priming' has been shown to be ATP-dependent and to involve the synthesis of PtdIns(4,5)P_2 (Eberhard *et al.* 1990, Hay & Martin 1993, Hay *et al.* 1995). Purification of the cytosolic components required for ATP-dependent priming has led to the identification of the PI-transfer protein (Hay & Martin 1993) and a PIP 5-kinase (Hay *et al.* 1995), but the PI4K that is needed for PtdIns(4,5)P_2 generation has never been identified. It is noteworthy that the first successful cloning of the type II PI4K resulted from the purification of a chromaffin granule-associated PI4K (Barylko *et al.* 2001). Whether this enzyme supports the process of exocytosis is yet to be determined.

The PI3K inhibitor, wortmannin (Wm), potently inhibits the degranulation caused by FcR activation in rat basophilic leukaemia (RBL) cells at concentrations that are consistent with inhibition of PI3K enzymes

(Yano *et al.* 1993). However, in other cells, such as pituitary gonadotrophs (Rao *et al.* 1997), pancreatic β cells (Straub & Sharp 1996) and PC12 cells (Oda *et al.* 1997), higher Wm concentrations were found to inhibit secretion, and, where kinetic analysis was performed, Wm selectively eliminated the sustained phase of secretion without affecting its early rapid phase (Rao *et al.* 1997). These effects of Wm (which were often attributed to MLCK inhibition by early studies) probably result from inhibition of type III PI4K enzymes that are also targets of Wm at the same high concentration range. Wm therefore appears to affect the process of replenishment of the readily releasable pool, at least in some cells. Moreover, a phenylarsine-oxide (PAO)-sensitive PI4K was identified as necessary for secretion in chromaffin cells (Wiedemann *et al.* 1996) and in pancreatic β-cells (Olsen *et al.* 2003). While PAO can have several targets other than PI4Ks, among the latter, PI4 KIIIα (another Wm-sensitive PI4K) shows the highest sensitivity to PAO (Balla *et al.* 2002). Recent evidence suggests that the small Ca^{2+}-binding protein, NCS-1, activates the Wm-sensitive PI4 KIIIβ enzyme and regulates priming and formation of the rapidly releasable vesicular pool in pancreatic β cells (Gromada *et al.* 2005). Similarly, reducing the levels of PI4 KIIIβ (Waselle *et al.* 2005) or PIP5KIγ (Gong *et al.* 2005, Waselle *et al.* 2005) has been shown to inhibit dense-core vesicle exocytosis. Recent studies have also indicated that PI3KC2α, by producing PtdIns(3)P, is also involved in neurosecretory granule exocytosis, adding 3-phosphorylated inositides to the list of potential regulators of the priming process (Meunier *et al.* 2005).

The regulatory role of phosphoinositides in synaptic vesicle release and recycling has also been well documented. Both synaptojanin-1, a phosphoinositide 5-phosphatase (McPherson *et al.* 1996), and PIP5KIγ (Wenk *et al.* 2001) have been shown to associate with synaptic vesicles, and knockout studies have confirmed that these enzymes are essential for normal synaptic functions, and have a role at multiple steps in synaptic vesicle exocytosis and recycling (Cremona *et al.* 1999, Di Paolo *et al.* 2004). Of the PI 4-kinases, PI4KIIα was shown to associate with synaptic vesicles (Guo *et al.* 2003). On the other hand, the *Drosophila* homolog of NCS-1, originally named frequenin and described as a major determinant of synaptic development and plasticity (Pongs *et al.* 1993), is a major regulator of the PI4KIIIβ protein (Weisz *et al.* 2000), as well as its yeast homolog, Pik1p (Hendricks *et al.* 1999). This suggests that there is a link between PI4KIIIβ function and synaptic transmission. It would not be surprising if both type-II and type-III PI4Ks were found to be important enzymes at distinct steps in the complex process of synaptic vesicle exocytosis and recycling.

Regarding the role of phosphoinositides in exocytosis, two additional questions remain to be answered. The first is whether the phosphoinositides are required on the surface of the secretory vesicles or at the plasma membrane, or perhaps in both locations. Initial reports have indicated that PtdIns(4,5)P_2 is needed on the surface of the vesicles where the presence of the enzymes has been demonstrated (Martin *et al.* 1997). However, studies using the PLCδ1PH-GFP fusion protein to image PtdIns(4,5)P_2 distribution failed to detect this lipid on the vesicular surface (Holz *et al.* 2000), indicating instead a significant increase in PtdIns(4,5)P_2 at the contact sites in the plasma membrane associated with the vesicle fusion process (Holz *et al.* 2000, Aoyagi *et al.* 2005). A recent report has established that plasma membrane PtdIns(4,5)P_2 levels are a determinant of the size of the readily releasable pool in chromaffin cells (Milosevic *et al.* 2005). Nevertheless, these observations do not rule out additional function(s) of PtdIns(4,5)P_2 at the surface of secretory vesicles. Another question relates to the identity of the molecules that are the targets of PtdIns(4,5)P_2. CAPS-1 (Ca^{2+}-sensitive activator protein of secretion) has been identified as a crucial factor in DCV exocytosis that acts between docking and fusion (Walent *et al.* 1992). This protein was shown to be a PtdIns(4,5)P_2-binding protein (Loyet *et al.* 1998) whose recruitment to the plasma membrane depended on PtdIns(4,5)P_2 levels (Grishanin *et al.* 2004). Interestingly, while CAPS-1 has a central PH domain and PH domains generally serve as lipid-binding modules (Lemmon & Ferguson 2000), the CAPS-1 PH domain is not the principal lipid-binding site of the molecule (Grishanin *et al.* 2004). Other proteins have also been suggested as targets of phosphoinositides in regulated secretion. These include the Ca^{2+}-sensitive SNARE-regulator protein, synaptotagmin, which binds inositides with its C2B domain (Schiavo *et al.* 1996, Bai *et al.* 2004); the Mint proteins (Okamoto & Sudhof 1997); and Rabphilin (Chung *et al.* 1998). Synaptotagmins have key roles in the Ca^{2+}-triggered fusion process (Tucker & Chapman 2002), and their phosphoinositide binding has other functions than vesicle priming, again enforcing the idea that phosphoinositides contribute to the regulation of the secretory process in multiple ways.

Cellular lipid homeostasis and phosphoinositides

Although maintenance of cellular lipid homeostasis is a well-recognized function of all eukaryotic cells, traditionally only some of its aspects are subjects of endocrine research. This is in spite of the fact that lipid metabolism at the level of the whole organism is a central topic of endocrinology. The role or roles of phosphoinositides in the regulation of lipid synthesis and transport are not widely recognized, but recent findings suggest that these processes are also under the control of phosphoinositides. Studies on PH domains that recognize specific inositol lipids have revealed that a special subgroup of PH domains can bind PtdIns(4)P with remarkable specificity

(Dowler *et al.* 2000). Proteins whose PH domains belong to this subgroup are the oxysterol-binding protein (OSBP), the ceramide-transfer protein (CERT) (Hanada *et al.* 2003) and the FAPP1 and FAPP2 proteins (Godi *et al.* 2004). With the exception of the FAPP1 protein, which appears to be a truncated adapter protein, all of these are lipid-binding/transfer proteins. The functions of OSBPs and their related proteins (ORPs) are mostly unknown, but they contain a lipid-binding motif that binds oxysterols or other phospholipids and a PH domain for interaction with membrane phosphoinositides (Lehto & Olkkonen 2003, Olkkonen & Lehto 2004). Oxysterols are among the most potent regulators of the transcription of genes that contribute to cholesterol synthesis and transport (Brown & Goldstein 1974, Olkkonen & Lehto 2004). OSBP overexpression increases cholesterol biosynthesis in chinese hamster ovary (CHO) cells (Lagace *et al.* 1997), and depletion of cholesterol or addition of 25-OH cholesterol promotes the association of OSBP with the Golgi complex (Ridgway *et al.* 1992, Storey *et al.* 1998). Since cholesterol biosynthesis is largely controlled by the SREBP transcription factor, whose nuclear translocation depends on its proteolytic cleavage in the Golgi complex (Brown & Goldstein 1999), the regulation of SREBP vesicular transport from the ER to the Golgi complex is a crucial step in cholesterol homeostasis and one that is likely to be controlled by PI 4-kinase(s). However, little is known about the connection between OSBP proteins and cholesterol homeostasis, and the involvement of phosphoinositides in SREBP trafficking.

More is known about the recently identified CERT protein, which is essential for the transport of ceramide from the site of its synthesis in the ER to the Golgi, where its conversion to sphingomyelin takes place (Perry & Ridgway 2005). CERT also contains a PH domain that binds PtdIns(4)P, and a single-point mutation within the PH domain that eliminates PtdIns(4)P binding is sufficient to render CERT completely dysfunctional (Hanada *et al.* 2003). PtdIns(4)P, therefore, is emerging as an important lipid regulator of the synthesis of sphingomyelin via its participation in the control of ceramide transport between the ER and the Golgi. It is a fascinating question why sufficient amounts of ceramide cannot reach the Golgi via the highly dynamic vesicular transport process that exists between the two organelles. This also suggests that ceramide is excluded from the budding CopII vesicles destined for the Golgi, probably because it is bound to a yet unidentified ER protein. The FAPP2 protein also contains a putative glycolipid-binding domain, but its natural ligand-binding partner is not known. The role (if any) that FAPP2 plays in cellular lipid metabolism, and whether the effects of FAPP2 knockdown on trafficking are related to its lipid-transport function, are also not known (Godi *et al.* 2004, Vieira *et al.* 2005).

Another indication of the importance of PtdIns(4)P in phospholipid synthesis comes from yeast studies. Synthesis of the aminophospholipid, phosphatidylethanolamine (PE), via decarboxylation of ER-derived phosphatidylserine (PS), takes place either in the mitochondria or in Golgi membranes (Voelker 2005). PS, therefore, has to be transferred to those membranes in order to be decarboxylated, and genetic studies have shown that Stt4p (the yeast homolog of PI4KIII alpha) is a regulatory component of this process at the ER/Golgi (but not at the mitochondrial) site (Trotter *et al.* 1998). It is not yet known why the Stt4p kinase is needed for lipid transfer or whether it acts at the donor or acceptor membrane site. It also remains to be seen whether a similar regulation of aminophospholipid synthesis by PI 4-kinases or by other phosphoinositides is present in higher organisms.

Sec14p, the yeast PI/PC-TP, is as an essential component of the secretion process from the Golgi in *Saccharomyces cerevisiae*, and also functions in phosphatidylcholine (PC) metabolism and maintains DAG levels in the Golgi (Routt & Bankaitis 2004). There is an intimate relationship between Sec14p and PtdIns(4)P levels within the Golgi, since inactivation of the Sac1p inositol lipid phosphatase (Guo *et al.* 1999) can 'bypass' Sec14p defects (Whitters *et al.* 1993). Mammalian PITP proteins come in various forms: in addition to PITPα and -β, a highly homologous PITP module is found in some of the larger RdgB proteins that are homologs of the *Drosophila* retinal degeneration protein (Vihtelic *et al.* 1993). Two of the PITPs, the small PITPβ and large RdgBα1, are Golgi-localized proteins, and the latter was recently shown to be critical for Golgi morphology and function by controlling DAG levels (Litvak *et al.* 2005). In this regard, the lack of RdgBα1 in mammalian cells causes a defect similar to that seen in the yeast *SEC14*. The small PITPβ appears to be an essential gene in the mouse, but PITPα knockout mice are viable with prominent defects in the transport of re-esterified triglycerides from the ER in enterocytes and a similarly defective lipid handling of hepatocytes (Bankaitis *et al.* 2004). Reduced levels of PITPα are responsible for the early-onset neurodegeneration described in the vibrator mouse (Hamilton *et al.* 1997). PITPs are clearly an exciting group of proteins on the border of phospholipid metabolism and vesicular trafficking, and will surely surprise us with novel functions in the near future.

Nuclear receptors, nuclear signaling and inositol phospholipids

Steroid and thyroid hormones that bind to intracellular receptors and regulate the transcription of their target genes in the nucleus seem to defy the developments of phosphoinositide research. Although a fraction of almost all of the inositide kinases and PLCs have been shown to be present in the nucleus under certain conditions

(Payrastre *et al.* 1992, Manzoli *et al.* 2005), and a separate nuclear phosphoinositide system has long been postulated (Irvine 2003), the link between the latter and the transcriptional apparatus has not been forthcoming. This gap began to narrow when genetic studies in yeast revealed that two inositol polyphosphate (IP) kinases, Ipk1 and Ipk2, are involved in mRNA export, chromatin remodeling and DNA metabolism (Odom *et al.* 2000, Shears 2004). These enzymes are critically important for the synthesis of highly phosphorylated inositols, such as $InsP_5$ and $InsP_6$, and also for the production of the pyrophosphorylated PP-$InsP_4$ and PP-IP_5, but it is not known at this point which (if any) of the highly phosphorylated inositols serves as an active regulatory species. Recent studies showed that the inositol pyrophosphates also regulate telomere length (Saiardi *et al.* 2005, York *et al.* 2005). Most recently, inositol multikinase, the mammalian homolog of Ipk2, was found to function as a wortmannin-insensitive PI 3-kinase in the nucleus. This observation raises the question of whether the lipids or the soluble inositol phosphates are the most important regulatory products of the enzyme (Carroll *et al.* 2004). In a separate line of research, structural characterization of the transcription factor, TFIIH, unveiled the presence of a PH domain in the molecule (Gervais *et al.* 2004). Moreover, an inositide-binding PHD domain was described in the chromatin-associated protein, ING2 (Gozani *et al.* 2003). The transcription factor Tubby, which, when mutated in mice, causes obesity, insulin resistance and sensory deficits (Carroll *et al.* 2004), was also shown to interact within its carboxy-terminal domain with plasma membrane PtdIns(4,5)P_2. Only upon hydrolysis of the lipid does Tubby become free to translocate to the nucleus (Santagata *et al.* 2001). The recent discovery of nuclear orphan receptors that bind phospholipids (Li *et al.* 2005), including PtdIns(3,4,5)P_3, as ligands (Krylova *et al.* 2005) is the latest addition to a list of nuclear proteins potentially regulated by phosphoinositides. It is expected that this area of research will greatly expand in the near future, putting nuclear events on a par with membrane-associated signaling processes as regulatory targets of phosphoinositides.

Concluding remarks

These examples of inositol lipid-regulated processes contributing to the complex cellular responses to hormones and neurotransmitters represent only a limited selection from the multitude of actions of phosphoinositides. The purpose of discussing them in this context was to identify areas of interest and to facilitate the incorporation of phosphoinositides as important topics in endocrine research. Over the last decade, it has become increasingly clear that the molecular elements of the classical messenger cascades, such as receptors, G proteins, ion channels and 'effectors', such as PLCs and A- or G- cyclases, are actively organized within the cell in the relevant membrane compartments. Phosphoinositides are at least as important in the trafficking and organization of signaling complexes as they are in the rapid generation of intracellular messengers. It is not too difficult to foresee a great expansion of knowledge in this research area and that cellular endocrinology will be an active contributor to, as well as a beneficiary of, this expansion.

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